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14. ABSTRACT

Prostate carcinogenesis is closely linked to aberrant activation of Ras or Ras signaling pathways (*e.g.*, Raf-MEK, or PI_3K pathways). The incidence of activating PI_3K mutations in early and advanced prostate cancer, or loss of PTEN is very high. Increased expression of the Ras/Raf/MEK/ERK pathway has been associated with advanced prostate cancer, hormonal independence and a poor prognosis. We have demonstrated that, when aberrantly activated, Ras is lethal to the cell unless a survival pathway also initiated by Ras is active. This survival pathway requires PKC-δ. Unlike the classical PKC isozymes, PKC-δ is not required for cell survival, and its inhibition or down-regulation in normal cells and tissues has no significant adverse effects. Inhibition of PKC-δ in human and murine cells containing an activated Ras protein, however, initiates rapid and profound apoptosis. In this work, we are testing the *hypothesis* that inhibition or down-regulation of PKC-δ in human and murine models of prostate cancer with aberrant activation of Ras signaling will cause targeted cytotoxicity in these tumors.

15. SUBJECT TERMS
Prostate cancer; Ras; small molecule inhibitors; drug development

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INTRODUCTION:

Although activating point mutations of Ras in prostate cancer, are not common, prostate carcinogenesis, in particular, is closely linked to aberrant activation of Ras or Ras signaling pathways (*e.g.*, Raf-MEK, or PI_3K pathways). The incidence of activating PI_3K mutations in early and advanced prostate cancer, or loss of PTEN is very high. Increased expression of the Ras/Raf/MEK/ERK pathway has been associated with advanced prostate cancer, hormonal independence and a poor prognosis. Strategies have been devised to target various stages of Ras signaling, ranging from inhibiting protein expression *via* antisense oligonucleotides, to blocking post-translational modification with farnesyltransferase inhibitors, to inhibiting downstream effectors. Unfortunately, these have shown minimal if any activity in prostate carcinoma in clinical trials, or have been limited by toxicity. Because wild-type Ras and its downstream effectors are required for many critical cellular functions in normal cells, the therapeutic window for inhibiting Ras directly may be too narrow to exploit. Our novel alternative strategy would circumvent this limitation. We have demonstrated that, when aberrantly activated, Ras is lethal to the cell unless a survival pathway also initiated by Ras is active. This survival pathway requires PKCδ.[1] Unlike the classical PKC isozymes, PKCδ is not required for cell survival, and its inhibition or down-regulation in normal cells and tissues has no significant adverse effects. Inhibition of PKCδ in human and murine cells containing an activated Ras protein, however, initiates rapid and profound apoptosis. This molecular approach, targeting tumor cells containing a mutated oncogenic protein (and sparing normal cells), by altering a second protein or its activity, is sometimes termed "synthetic lethality."[2,3] Analogously, the dependency of tumor cells upon the activity of a non-oncogenic protein is sometimes termed "non-oncogene addiction." *Hypothesis*: inhibition or down-regulation of PKCδ in human and murine models of prostate cancer with aberrant activation of Ras signaling will cause targeted cytotoxicity in these tumors.
The Specific Aims of this Idea Proposal are: i.)Test the hypothesis that inhibition or down-regulation of PKCδ in human prostate cancer cell lines with dysregulation of Ras pathways selectively induces apoptosis. Using molecular modeling, multiple analogs of the current lead PKCδ inhibitor have been predicted to have more specificity and higher potency that the current lead compound. A collaboration with a leading medicinal chemist produced one analog with superior pharmacokinetics. Thirty six next generation other analogs have been synthesized and tested for activity and isozyme specificity *in vitro* and in tissue culture. The best one or two analogs will then be tested *in vivo* (below) in a head-to-head comparison with the current lead compound to identify an optimal PKCδ inhibitor. ii.) Determine whether constitutive activation of *selected Ras effector pathways alone* (PI_3K Pathway, *via* the commonly-occurring loss of PTEN or activating mutations in PIK_3CA [p110α]; or constitutive, aberrant activation of the MEK-ERK signaling pathway) is sufficient to make prostate cancer cells susceptible to apoptosis after PKC-δ inhibition. iii.) Test the ability of PKC-δ inhibitors to induce selective cytotoxicity in human *prostate cancer stem cells*. iv.) Test this targeted approach in *in vivo* models of human prostate carcinoma. A xenograft model will be employed, utilizing an activating Ras-mutant human prostate carcinoma cell line and a human prostate carcinoma cell line with aberrantly-activated Raf-signaling.

Innovation: Ras signaling is an attractive target for therapy of prostate cancer, but approaches aimed at Ras itself, or its critical signaling pathways, which are required in normal tissues, have had limited success. This "non-oncogene addiction" approach, however, exploits a weakness of

tumor cells with aberrant activation of Ras or Ras effectors – their absolute requirement for a survival pathway mediated by PKC-δ. In contrast, normal cells and tissues do not require PKC-δ.
Impact: Current therapies for prostate cancer are inadequate, and aberrant activation of Ras or Ras pathways are common. A novel therapeutic modality selectively targeting prostate cancers with activation of Ras or Ras pathways would make a significant impact on the way prostate cancer is treated.

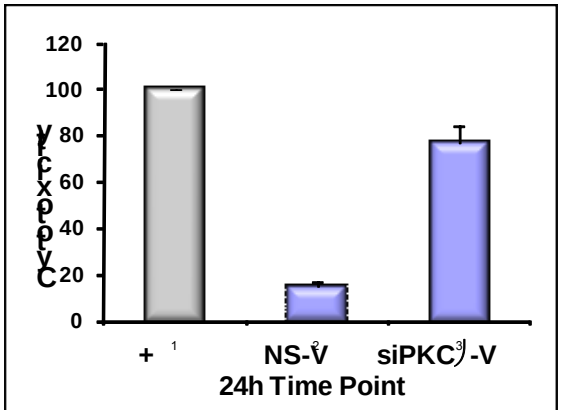

Fig. 1: Human prostate cancer cells were infected with lentiviral vector containing a non-specific shRNA (NS-V) or a PKCδ-specific shRNA (siPKCδ-V), and an LDH release cytotoxicity assay was carried out at 24 hrs. "+" represents the maximum total release of LDH from cells artificially lysed, according to manufacturer's instructions. Background release was subtracted from the values. Error bars indicate SEM.

BODY:

TASK 1: Testing human prostate cancer cell lines for sensitivity to PKCδ inhibition

Status: IN PROGRESS

Methods: Using cancer cell lines with known activating mutations in H-Ras, comparing these with human prostate cells containing wild-type Ras alleles: (non-Ras-mutated) prostate epithelial cells

Task 1a) Using siRNA to suppress PKCδ
Task 1b) Using new, specific small-molecule PKCδ inhibitors.

- Verify their PKCδ inhibitory activity and isozyme-specificity will be verified *in vitro* using purified PKC isozymes
- Testing their ability to induce apoptosis in prostate cancer cell lines, and selection of the most potent and PKCδ isozyme-selective for *in vivo* testing.

Assays: MTS assay for enumeration of cells at 48 and 72 hrs after treatment. LDH release assays or flow cytometry assays to assess cytotoxicity

Results:

Task 1a: **siRNA –** To demonstrate the specificity of this targeted approach, we first used PKCδ-specific lenti-viral based shRNA to efficiently knockdown PKCδ protein in a human prostate cancer cell line, DU145. We developed lentiviruses containing shRNA directed against PKCδ, or a scrambled shRNA. Viruses were titered to determine moi for use. They were then used to infect prostate cancer cell lines. Even within 24 h, we observe very significant cytotoxicity, as assessed by LDH release (see **Fig. 1** for representative experiment). Parallel studies using these lentiviral vectors in other cell lines, including normal prostate cancer cells, are underway to validate the studies described below, which show marked sensitivity of a variety of prostate tumor cells to PKCδ inhibitors.

Task 1b. We will first describe the development of new specific PKCδ inhibitory molecules, and then show the results of the testing of these compounds on prostate cancer cell lines.

Pharmacophore Modeling and Development of new PKCδ Inhibitors: Highly isotype-specific PKCδ-inhibitory small molecules had not been identified by others to date. With our discovery and genetic validation that PKCδ is the specific target molecule for this Ras-targeted approach, we generated a pharmacophore model based on molecular interactions with "novel" class PKC isozymes. We established a initial pharmacophore model for PKCδ inhibitors, using mallotoxin/rottlerin [Lead Compound 1 (**LC-1**)] as a prototype structure for a moderately PKCδ-specific inhibitor (IC_{50}=5μM), and incorporated protein structural data for PKCε, another member of the "novel" group of PKC enzymes, which is also inhibited by mallotoxin. LC-1 is a naturally-occurring product, with moderate aqueous solubility, and oral bioavailability.[4] It inhibits purified PKCδ at an IC_{50} of 3-5 μM *in vitro*, and inhibits PKCδ in cultured cells with an IC_{50} of 5 μM *in vivo* (but at 0.5 μM with exposure for >24 hrs, because of down-regulation of the PKCδ protein[5]). It is relatively selective for PKCδ over PKCα (PKCδ IC_{50}:PKCα IC_{50} is approximately 30:1). Furthermore, as we have published, this compound not only directly inhibits purified PKCδ, but also, over longer periods of exposure, significantly down-regulates PKCδ protein specifically, while having no effect on the levels of other PKC isozymes.[5] Thus, this compound inhibits PKCδ at two levels. We have demonstrated "Ras-specific" activity of this compound in a number of publications and assays (see above). Daily *i.p.* doses of up to 40 mg/kg (800 μg/20 g) in mice do not produce any overt toxicity in our xenograft studies or others.[4] Stability: Informal stability testing demonstrates >95% stability as a powder at room temp for >6 months. Toxicology: Pilot and published toxicity data indicate that the compound has a low toxicity profile (lowest lethal dose = 750 mg/kg, rat oral); 120 mg/kg (oral 6-day rat study) is the lowest toxic dose.[6,7] This relative safety, combined with its *in vivo* efficacy, makes Lead Compound I attractive as a starting point for modification and drug development. We have demonstrated that better therapeutic candidates can be developed from it. The rationale for the development of new inhibitors was to improve the PKCδ-selectivity and potency. [Potential limitations on LC-1 itself as a therapeutic agent (despite its *in vivo* safety and activity) include its lack of high specificity for PKCδ; its off-target effects, including inhibition of Cam Kinase III, MAPKAP-K2, and PRAK1 at IC_{50}s of <10 μM; its non-PKC-mediated effects on mitochondrial uncoupling and modulation of death receptor pathways;[8,9] and the lack of composition-of-matter IP around it, which would preclude eventual clinical development by big pharma.]

We designed and synthesized a 2nd generation set of analogs. In Analogs 1 and 2, the "head" group (A) was been made to resemble that of staurosporine, a potent general PKC inhibitor and other bisindoyl maleimide kinase inhibitors, with domains B and C conserved to preserve isozyme specificity. Ease of synthesis was a major factor in the design of this head group. Analogs 3 to 5 have "head groups" from other known kinase inhibitors: 1) Analog 3 from the crystal structure of an inhibitor bound to CDK2 (pdb code: 1FVT); 2) Analog 4 based on purine, found in a number of different potent kinase inhibitors; and 3) Analog 5 from a potent inhibitor of aurora kinase (pdb code 2F4J). The first 2nd generation chimeric molecule, KAM1, was indeed active, and more PKCδ-specific (see **Table 2,** below), and showed activity against cancer cells with activation of Ras or Ras signaling.[10] Another 2nd generation compound we generated (CGX, with a very different composition but which fit the pharmacophore model) has demonstrated activity against multiple human cancer cell lines with activated K- or H-Ras alleles *in vitro* and *in vivo* in animal models. On the basis of SAR analysis of KAM1, we have this year generated 36 new 3rd generation compounds.

The PKCδ inhibitory activity and isozyme-specificity of the 36 3rd generation analogs was

assayed *in vitro*, using recombinant PKC isozymes, prior to comparative testing on prostate cancer cell lines.

Method: These assays utilize fluorogenic FRET detection (Z-lyte, R&D Systems) technology and peptide substrates, are robust and validated, and have been used to screen the 2nd and 3rd generation PKCδ inhibitors we have synthesized.

Results:

1. PKCδ Activity Assays of 3rd Generation Compounds

Recombinant PKCδ enzyme and FRET substrate. Compounds were tested at 5, 10 and 50 μM. (**Figs. 1-4**)

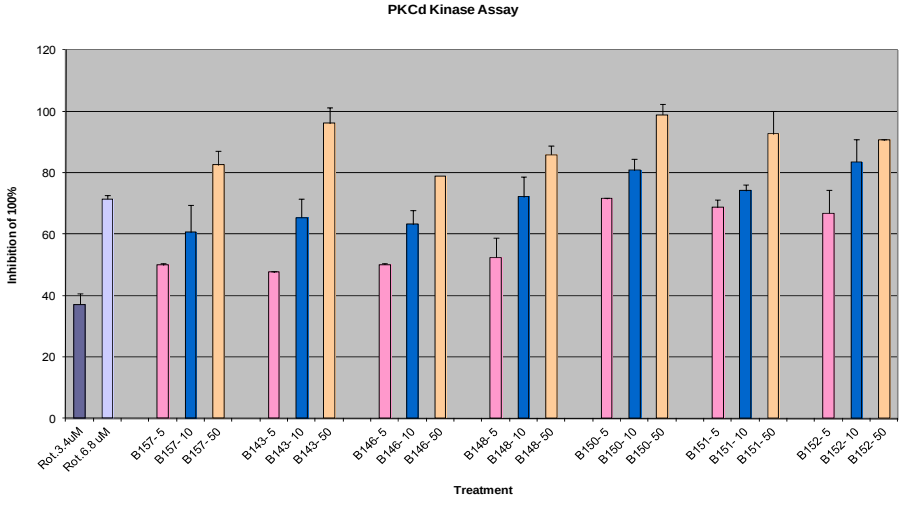

Fig. 1: PKCδ activity, expressed as % inhibition. Compounds tested at the concentrations indicated (μM).

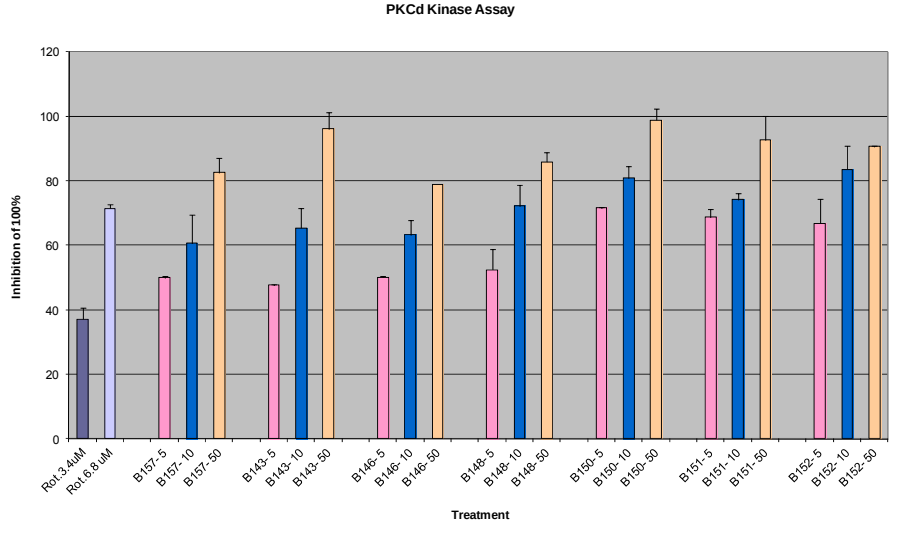

Fig. 2: PKCδ activity, expressed as % inhibition. Compounds tested at the concentrations indicated (μM).

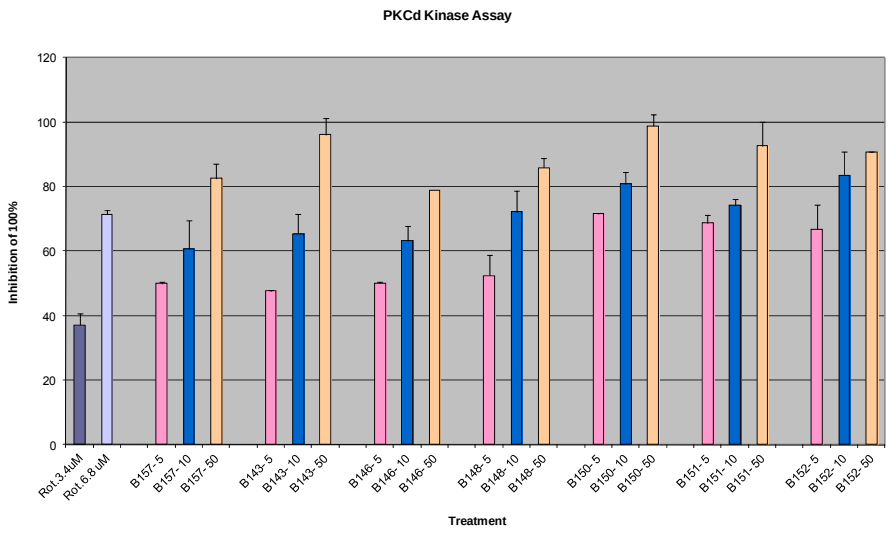

Fig. 3: PKCδ activity, expressed as % inhibition. Compounds tested at the concentrations indicated (μM).

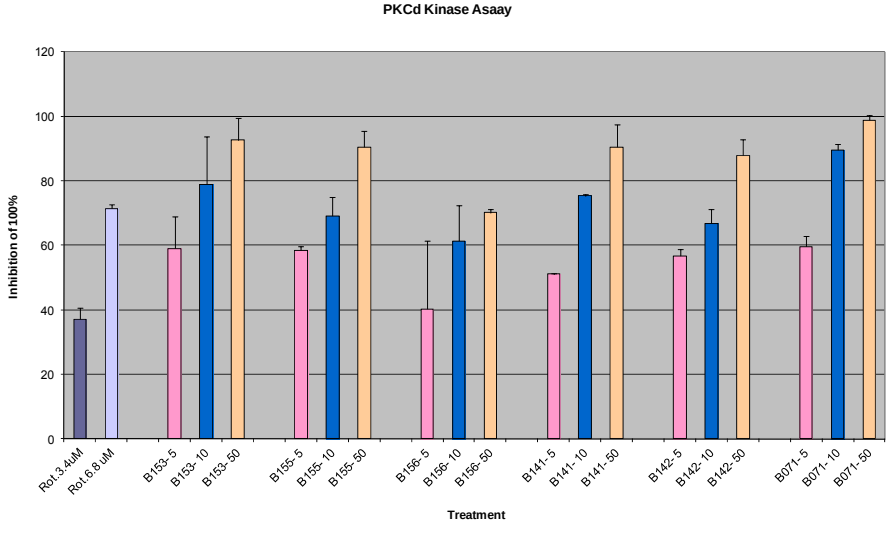

Fig. 4: PKCδ activity, expressed as % inhibition. Compounds tested at the concentrations indicated (μM).

Compounds with the highest inhibitory activity (106, 109, 111, 125) were too potent to allow assessment of IC50s in the assays above, and the assays were repeated using lower concentrations of the inhibitors (**Figs. 5-6**).

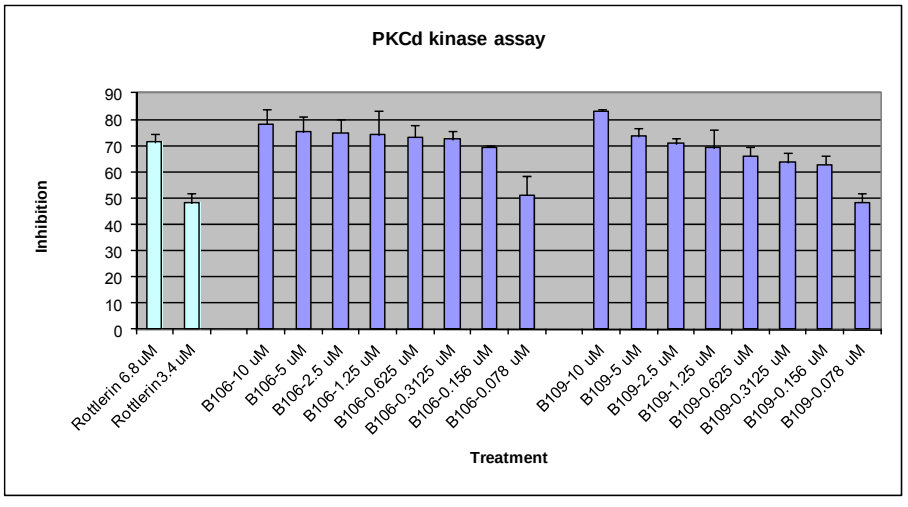

Fig. 5: PKCδ activity, expressed as % inhibition. Compounds tested at the concentrations indicated (μM).

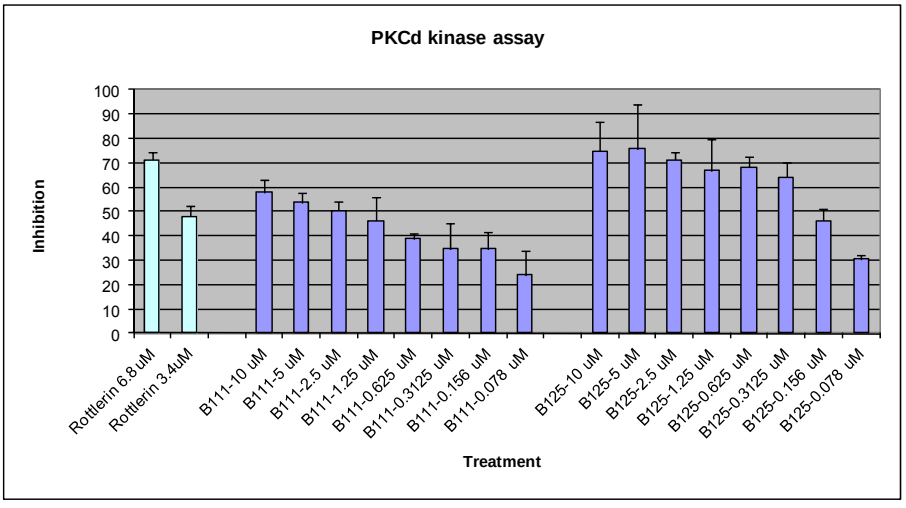

Fig. 6: PKCδ activity, expressed as % inhibition. Compounds tested at the concentrations indicated (μM).

2. PKCα Activity Assays of Selected 3rd Generation Compounds

Recombinant PKCα enzyme and FRET substrate were utilized. A standard curve is shown in **Fig. 7**. Selected compounds were tested at 5, 10 and 50 μM. (**Figs. 8-9**)

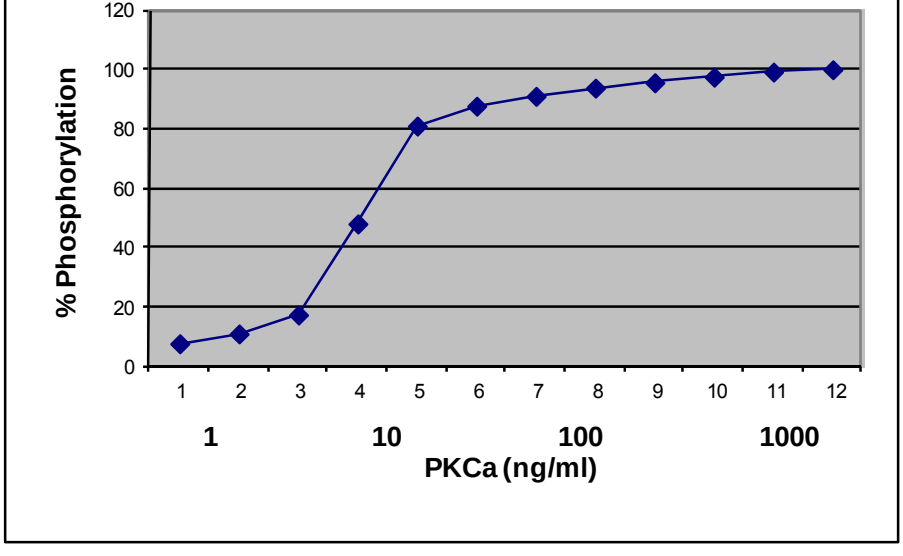

Fig. 7: PKCα standard curve.

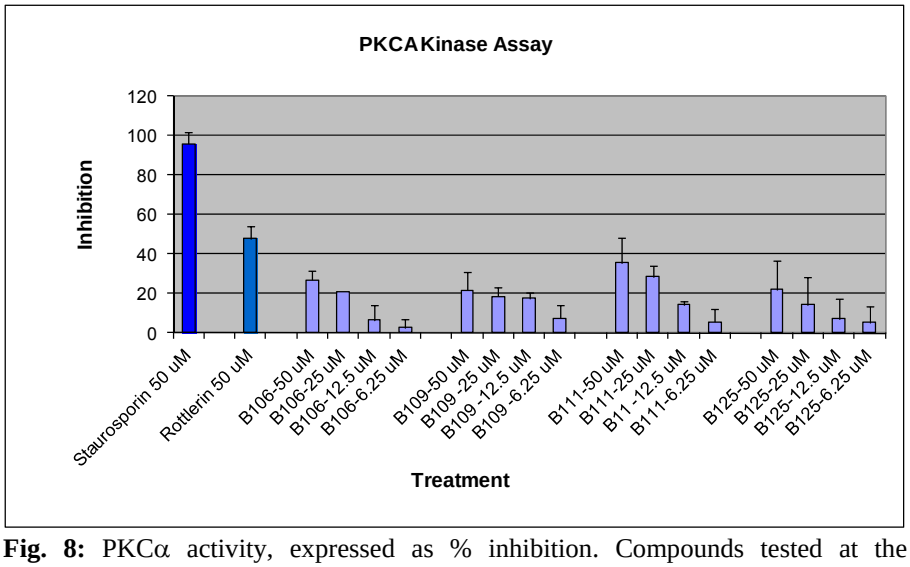

Fig. 8: PKCα activity, expressed as % inhibition. Compounds tested at the concentrations indicated (μM).

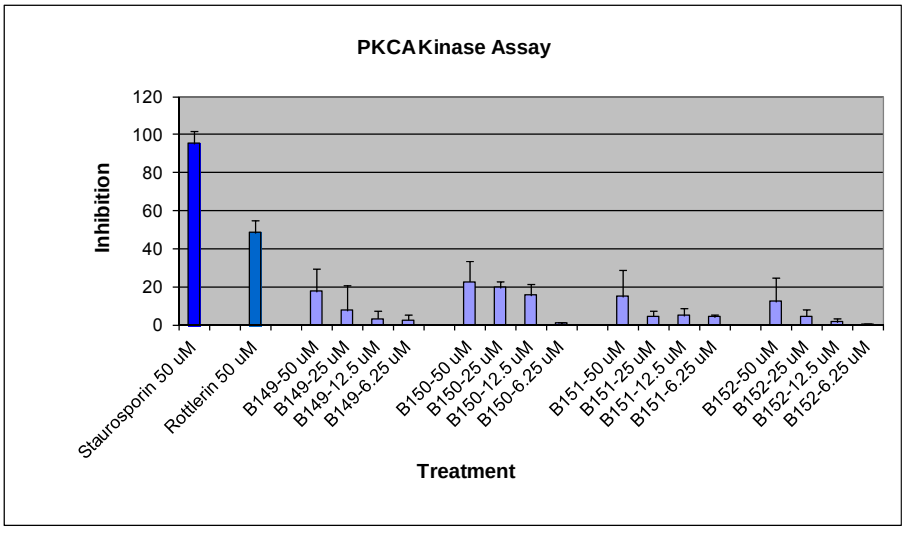

Fig. 9: PKCα activity, expressed as % inhibition. Compounds tested at the concentrations indicated (μM).

The data generated from replicates of the types of assays shown above were plotted for comparison (**Fig. 10**).

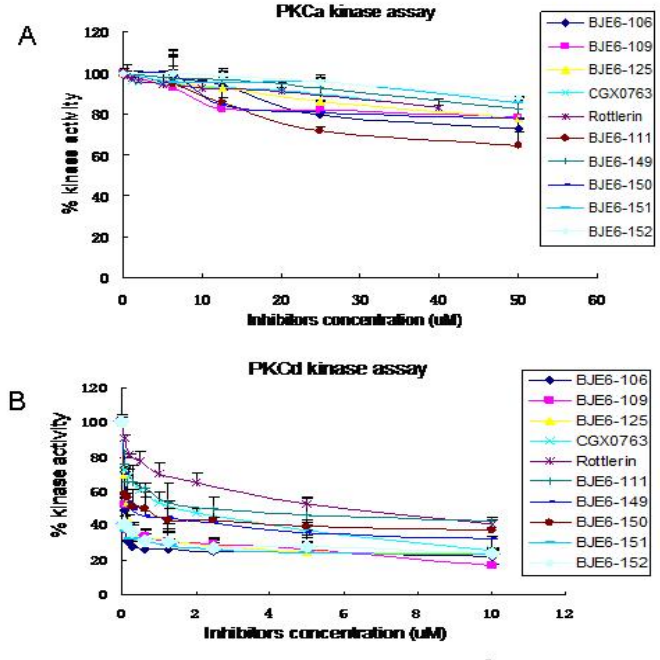

Figure 3. Inhibition effects of new PKC δ inhibitors on PKC isotopic kinase activity assays. (A). Kinase assay for their ability to inhibit PKC α activity. **(B).** Kinase assay for their ability to inhibit PKC δ activity. Activity curves showed that these inhibitors selectively inhibit PKC δ but PKC α.

Fig. 10: Plots of PKCα activity (A) and PKCδ activity (B), expressed as % control enzyme activity. Compounds tested at the concentrations indicated (μM).

The information from the enzymatic activity/inhibitor assays above were compiled into a summary table (**Table 1**) for purposes of comparison.

Compound	PKCδ IC50	PKCα IC50	Cytotoxicity relative to Rottlerin DU145	HRasG12\	NP-PCSC	P-PCSC
Rottlerin	4.4		xx	xxx	xxxx	xx
KAM-1	5.6					
B058	5.8		o	o	o	x
B071	3		o	o	o	o
B095	8		x	o	o	xx
B097	9.5		xx	o	o	o
B106	0.08	200	xxx	xxx	xxxx	
B108	3		o	o	o	o
B109	0.09	800	o	o	o	o
B111	2.5	100	x	o	o	
B112	6		xxx	xx	xx	xx
B117	4.5		o	o	o	o
B118	? 10		o	o	o	o
B121	4		o	o	o	o
B125	0.25	800	x	o	o	x
B128	6.5		xxx	o	o	xx
B129	6.5		x	o	o	x
B130	4		o	o	o	o
B131	10		o	o	o	o
B136	14		x	o	o	o
B137	3		o	o	o	o
B141	4.8		x	o	o	o
B142	3.5		o	o	o	o
B143	6		o	o	o	o
B146	5		o	o	o	o
B147	5.8		x	xx		x
B148	4		o	o	o	o
B149	0.31	>1000	x	xx	xx	o
B150	0.625	800	o	o	o	o
B151	< 0.05	>1000	o	o	o	o
B152	< 0.05	>1000	o	o	o	o
B153	3		o	o	o	o
B154	23		o	o	o	o
B155	3		o	o	o	o
B156	7.5		o	o	o	o
B157	5		o	o	o	o
B158	4.5		o	o	o	o
B159	7		xxxx	xx	xx	xx

Table 1: Summary PKCδ and PKCα inhibitory activity of 36 3rd generation compounds, expressed as IC50. Summary of relative cytotoxicity on multiple prostate cancer cell lines, relative to rottlerin.

Interpretation: Certain of the 3rd generation compounds show substantially greater PKCδ inhibitory activity and specificity than LC-1 or 2nd generation compounds. For example, one such novel compound ("B106") is much more potent than LC-1 (**Table 1**), producing substantial cytotoxicity against Ras-mutant tumor lines at concentrations ~40 times lower than LC-1. This compound is also active *in vivo*, in a Ras-mutant cell xenograft assay. Both LC-1 and B106 dramatically inhibited clonogenic capacity of Ras-mutant tumor cell lines after as little as 12 h exposure. A newer derivative of this particular compound (CGD63), not yet optimized with respect to drug-like properties, has a PKCδ IC_{50} in the range of 0.05 μM (compared to 3 μM for LC-1), is 1000-fold more inhibitory against PKCδ than PKCα *in vitro*, and produces cytotoxic activity against Ras-mutant cells at nM concentrations. (Specificity for PKCδ over classical PKC isozymes, like PKCα, is important: inhibition of PKCα is generally toxic to all cells, normal and malignant, and would make our agent non-"tumor-targeted.") We are therefore seeking to maximize PKCδ-isozyme-specificity for the inhibitors to retain the tumor-targeted cytotoxic properties. We will eventually test selected inhibitors against an entire panel of recombinant PKC isozymes, including the classical, novel and atypical classes.

Table 2 compares the 3 generations of PKCδ-inhibitory compounds tested to date.

Table 2: Comparison of PKCδ-inhibitors

Generation	PKCδ IC_{50}	PKCα IC_{50}	PKCδ/PKCα Selectivity	*in vitro* Ras-specific Cytotoxicity	*in vivo* Ras-specific Cytotoxicity
1	3 μM	75 μM	28-fold	3 μM	Yes
2	2 μM	157 μM	56-fold	3-5 μM [b]	Yes [b]
3 [a]	0.05 μM	50 μM	1000-fold	<0.5 μM [b]	Not tested

[a] not all 3rd generation compounds have been extensively tested. This is data from one of the most active 3rd gen compounds to date.
[b] not yet optimized for drug-like properties, so *in vitro* activity represents a minimum of the potential activity.

Future Plans for Synthetic Strategy and Approach: A major goal of the next generation synthesis will be to *increase the drug-like properties of the drug candidate molecules*, as the 3rd generation molecules have not yet been optimized for drug-like properties (*e.g.*, improved water solubility; stability; ease of formulation; oral bioavailability and favorable toxicity profile). We will start by simply adding polar groups to the B106 scaffold, which is thus far the most promising analog. R1 and R2, which are hydroxyl groups in LC-1 and are hydrogen atoms in B106, will be sequentially substituted with OH groups which should improve water solubility. In addition, we plan to perform an isosteric replacement of the aromatic CH groups with basic nitrogen atoms which will be protonated at physiological pH providing for additional water solubility and perhaps improved potency. Based on the biological activity of these 4th generation of analogs, our SAR will be further guided by these outcomes. In addition, we plan to make the cap group from the staurosporine scaffold, more similar to the natural staurosporine structure with the ultimate goal of preparing the initial chimeric analog series. Space does not permit a detailed description of the synthetic plan but it can be said that these new 4th generation analogs do not pose a significant synthetic challenge and should be amenable to the basic synthetic chemistry platform that was developed to make KAM1.

3. Testing of 3rd Generation PKCδ Inhibitor Compounds in Prostate Cancer Cell lines

Materials and Methods:

- Cells were grown on 60mm tissue culture dishes, seeded to 1 x 10E5 cells per well:
- Cells were allowed to grow 24 hrs at 37oC and 5% CO2.
- On treatment day, media was removed from each plate and replaced with either vehicle or test compound in growth media
  - DMSO (vehicle for compounds)
  - Compounds tested at various concentrations
- At 48 or 72 hrs, cells were harvested, and viable cell mass quantitated via MTT or MTS assay.

We have tested the entire panel of 36 3rd generation compounds against a prostate cancer cell line with an activating Ras mutation. The compounds were prepared in stock solutions as shown in **Table 2**. Results from representative cytotoxicity assays are shown below (**Figs. 11-15**)

	Molecular formula							
BJE6	C	H	N	O	FW	Weight (mg)	Mols (10 or 40mM stock)	Vol of diluent (ml) for Mols
-112	33	27	1	2	469.5730	8.5	0.01	1.810
-128	33	25	1	2	467.5571	9.8	0.01	2.096
-129	33	29	1	2	471.5889	6.4	0.01	1.357
-136	20	17	1	1	287.3551	7.6	0.01	2.645
-154	32	31	1	1	445.5947	4.0	0.01	0.898
-159	24	21	1	2	355.4291	7.2	0.01	2.026
-117	33	27	1	2	469.5730	30.4	0.04	1.618
-118	33	29	1	2	471.5889	45.5	0.04	2.412
-125	23	21	1	2	343.4184	24.9	0.04	1.813
-137	31	25	1	1	427.5363	17.6	0.04	1.029
-141	21	17	1	2	315.3652	50.3	0.04	3.987
-142	22	19	1	2	329.3918	75.3	0.04	5.715
-143	30	27	1	1	417.5415	36.6	0.04	2.191
-146	32	27	1	1	441.5629	33.8	0.04	1.914
-147	21	19	1	0	285.3823	12.6	0.04	1.104
-148	22	21	1	0	299.4089	30.7	0.04	2.563
-149	21	19	1	1	301.3817	14.2	0.04	1.178
-150	22	21	1	1	315.4083	29.3	0.04	2.322
-151	32	29	1	2	459.5782	34.7	0.04	1.888
-152	23	21	1	1	327.4190	44.4	0.04	3.390
-153	31	29	1	1	431.5681	28.3	0.04	1.639
-155	21	19	1	0	285.3823	52.4	0.04	4.590
-156	22	21	1	0	299.4089	55.4	0.04	4.626
-157	30	29	1	1	419.5574	25.9	0.04	1.543
-158	32	31	1	1	445.5947	40.8	0.04	2.289

Aliquot procedure:
1-Prep & label 15ml tubes
2-Dispense vol fo DMSO to labeled tubes
3-Take ~1ml out of tube and resuspende powder in vial
4-Transfer resuspended DMSO from vial to tube, rinse out vial with DMSO in tube.

Table 3

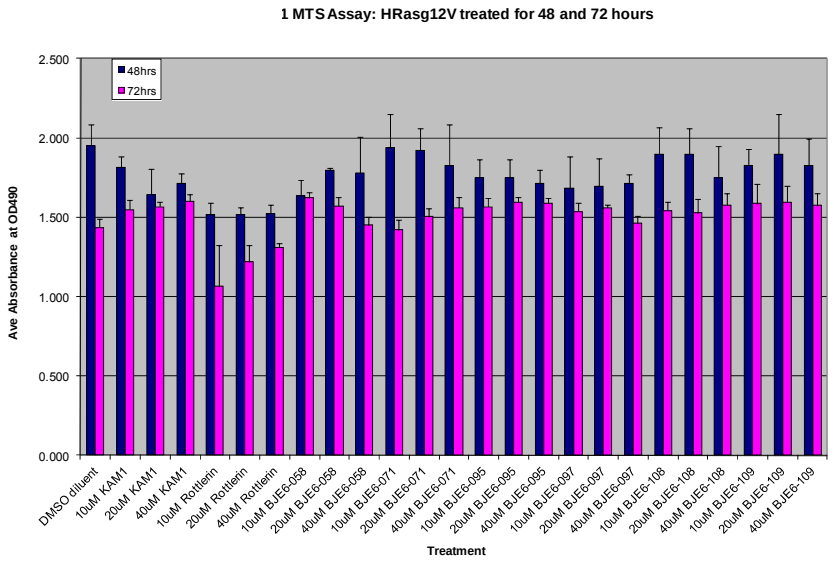

Fig. 11: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

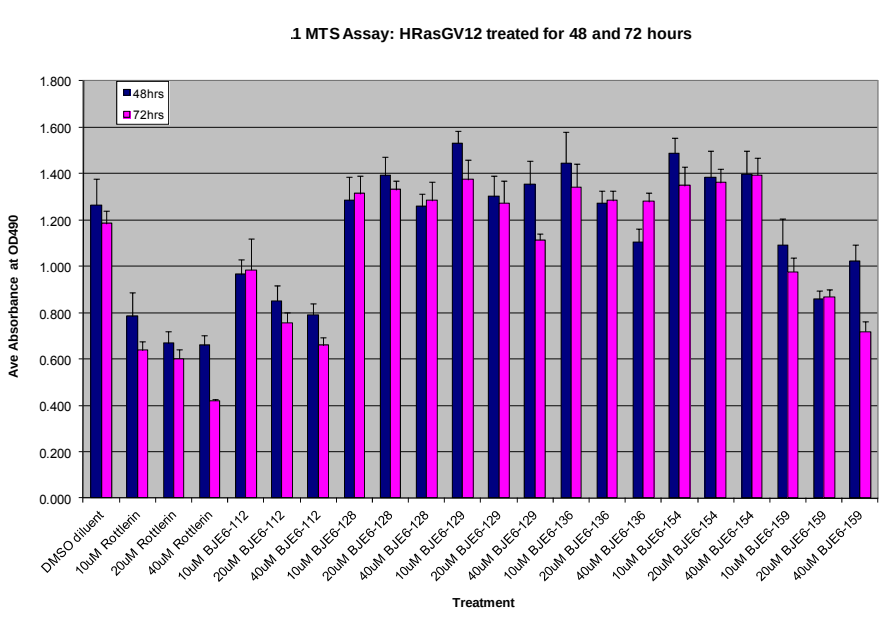

Fig. 12: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

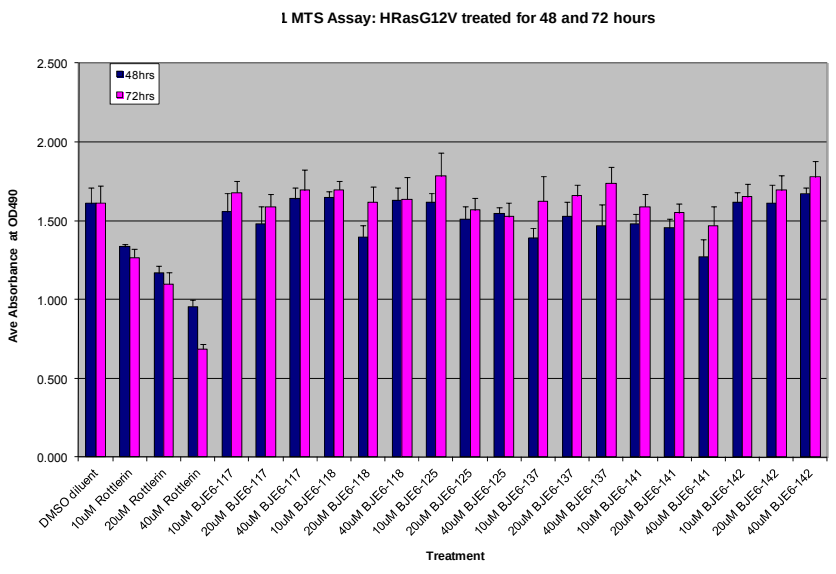

Fig. 13: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

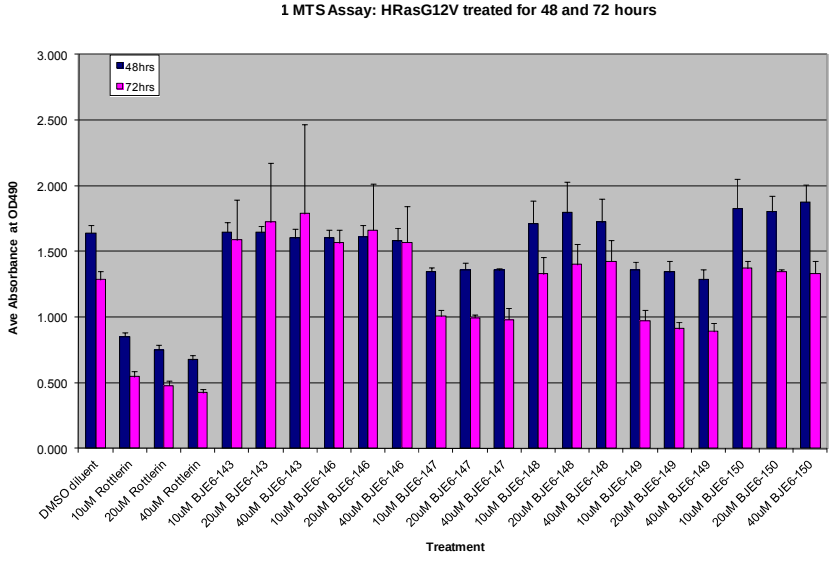

Fig. 14: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

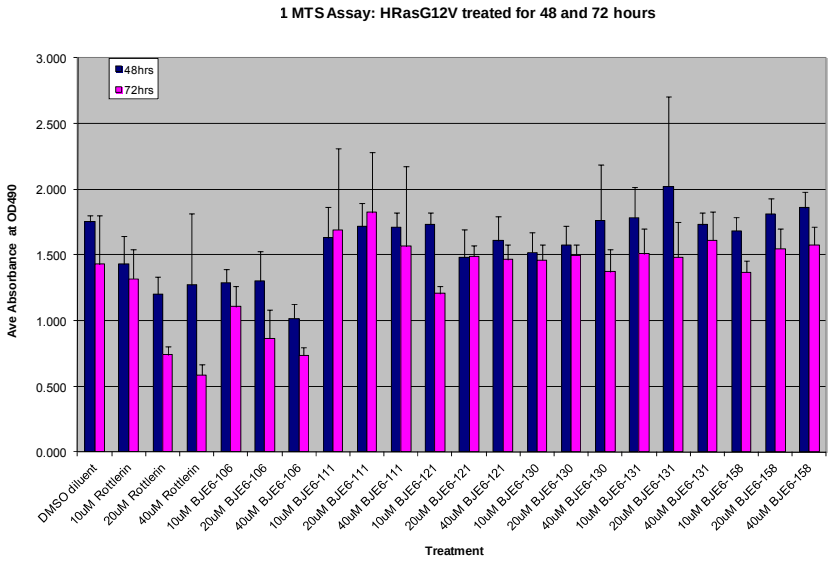

Fig. 15: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

Interpretation: Certain 3rd generation compounds (106, 147, 149, 112 and 159) show toxicity against this cell line comparable to LC-1 or greater than LC-1.

Task 1c) Determine the duration of PKCδ inhibition required to irreversibly initiate the apoptotic process.

Method/Assays:

1. Washout Studies: Exposure to inhibitors of PKCδ for different intervals of time, followed by washout, and assay of cell number over time. In this representative study, the DU145 cell line was used, and LC-1 (rottlerin) was used as the inhibitor (**Fig. 16A**).

2. Clonogenic Assays: Human prostate cancer stem cells in culture were exposed to a small-molecule inhibitor of PKCδ for 6, 18, 24, or 48 hrs, then the inhibitor was washed out and a clonogenic assay carried out. Colonies formed were enumerated. Treatment times indicate the duration of exposure to the inhibitor prior to replating (**Fig. 16B**).

Interpretation: Cytotoxic effects on prostate cancer cells are observed after exposure to PKCδ inhibitors for a period of 6 hrs. Longer periods of exposure produce progressively more toxicity. Replacing with fresh PKCδ inhibitors does not enhance the cytotoxic effect. These studies will be repeated using selected 3rd or 4th generation inhibitors.

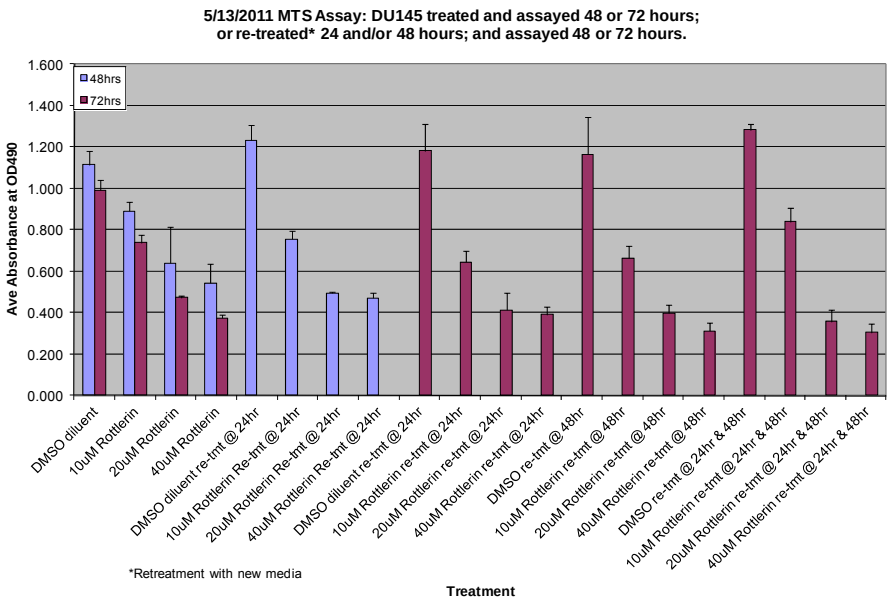

Fig. 16A: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

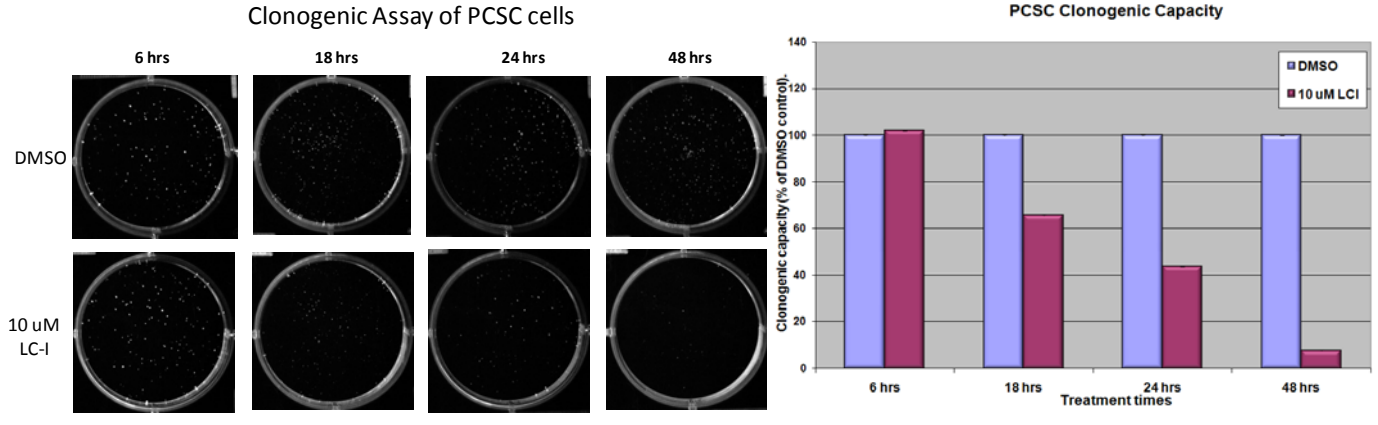

Fig. 16B: Clonogenicity Assay using human Prostate Cancer Stem Cells (PCSC). Human prostate cancer stem cells in culture were exposed to a small-molecule inhibitor of PKCδ for 6, 18, 24, or 48 hrs, then the inhibitor was washed out and a clonogenic assay carried out. Treatment times indicate the duration of exposure to the inhibitor. Error bars (very small and difficult to see) indicate SEM. $p < 0.05$ for the 18, 24 and 48 hr exposures compared to DMSO control.

TASK 2: Determine whether constitutive activation of *selected Ras effector pathways alone* is sufficient to make human prostate cancer cells susceptible to apoptosis after PKCδ inhibition: (utilizing prostate cancer cells with aberrant activation of the PI_3K pathway or aberrant activation of the Ras-MEK-ERK pathway.

Status: IN PROGRESS

Task 2a) PI_3K Pathway: utilizing the LNCaP line and prostate cancer lines with the commonly-occurring loss of PTEN [*e.g.*, DU145] [11]

Progress:

We have tested the entire panel of 36 3rd generation compounds against a prostate cancer cell line with activation of PI_3K pathway. Examples of such studies are shown below (**Figs. 17-21**). Several of the most active compounds were then compared head-to-head (**Fig. 22**).

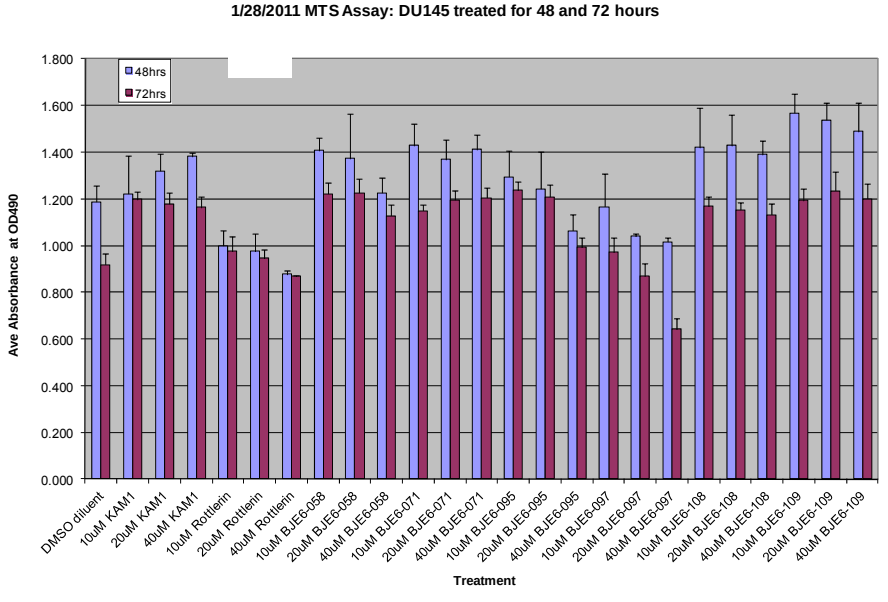

Fig. 17: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

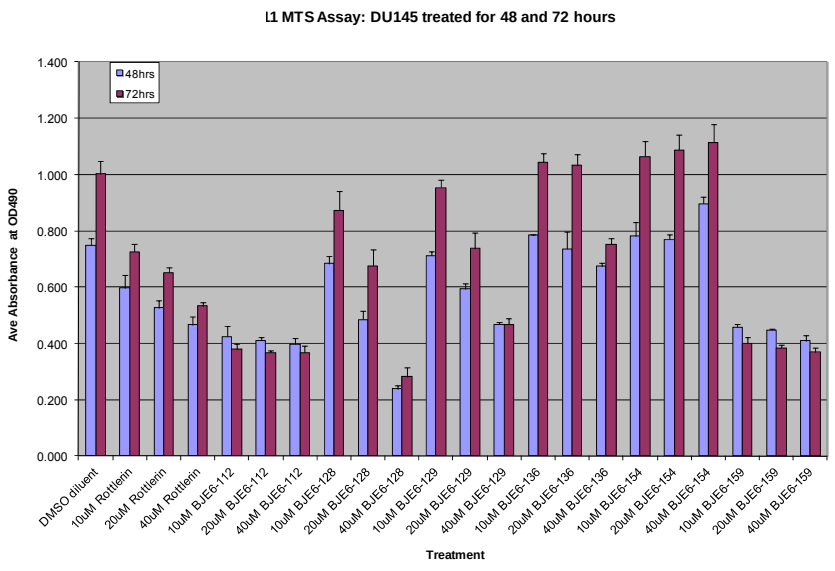

Fig. 18: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

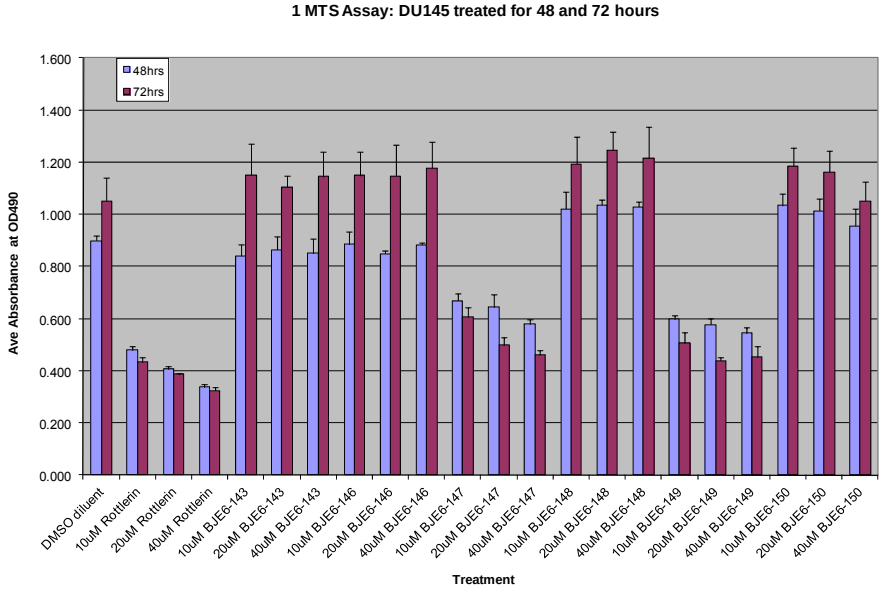

Fig. 19: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

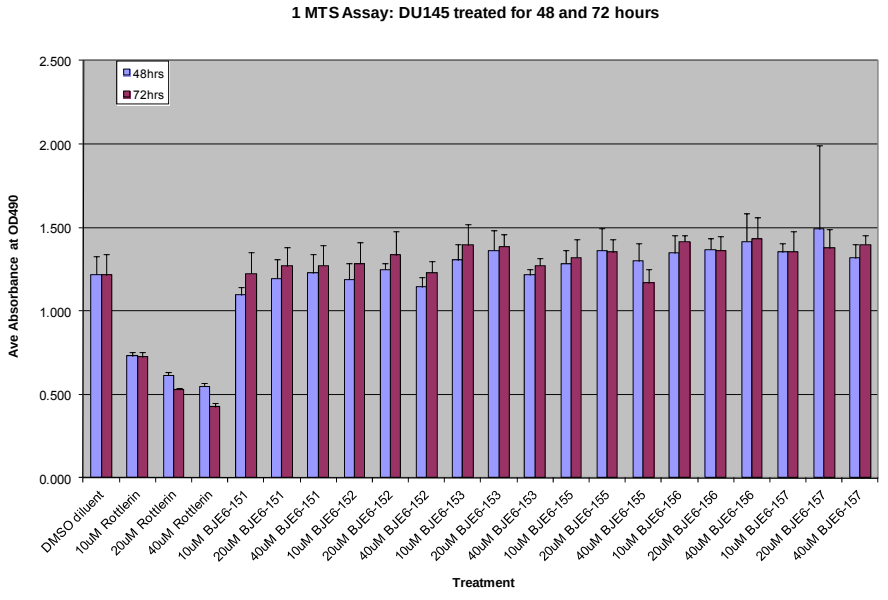

Fig. 20: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

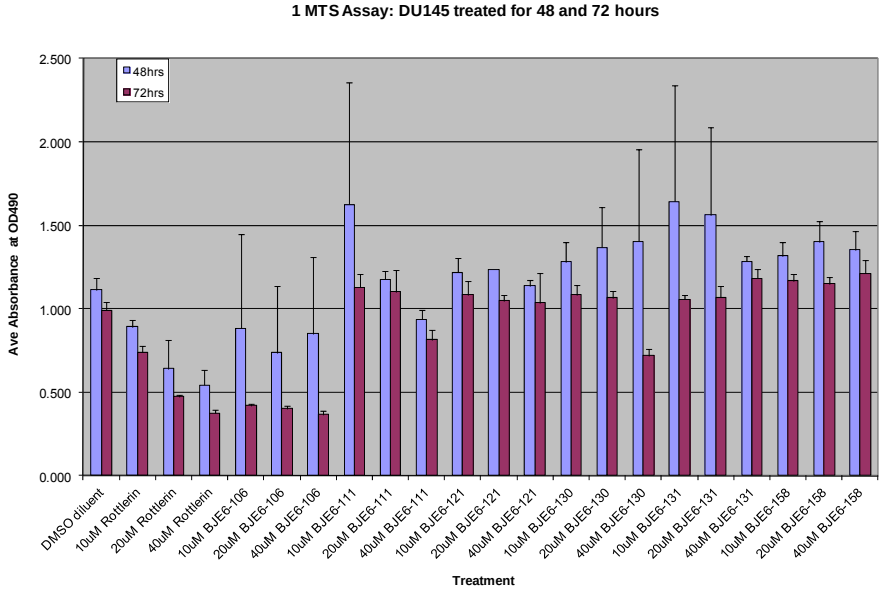

Fig. 21: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

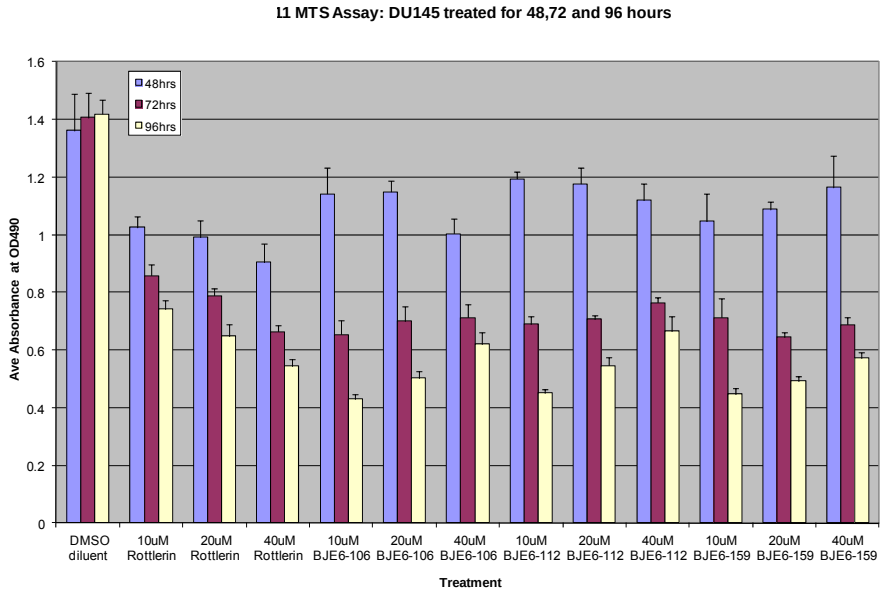

Fig. 22: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

Interpretation: Certain 3rd generation compounds (106, 112, and 159) show toxicity against this cell line comparable to LC-1 or greater than LC-1). Interestingly, cytotoxicity did not always correlate with *in vitro* PKCδ inhibitory activity. (*e.g.*, the highly potent B109 had no activity in culture. We hypothesize that this is due to the highly-hydrophobic nature of some of these compound, inhibiting their entry into cells. Our next generation molecules will be optimized for drug-like properties to overcome this problem.

Task 2b) MEK-ERK Pathway: Human prostate cancer cell line CWR22Rv1 has constitutive, aberrant activation of the MEK-ERK signaling pathway, with wild type PTEN and PI_3K signaling

Progress: The analysis of effects of PKCδ inhibitors on CWR22Rv1 has not yet begun, as we are still determining the most potent and specific PKCδ-inhibitory compounds to be used for testing. We will only test the most potent compounds on the cells rather than extensive testing of the full current panel of 36 compounds, some of which are poor PKCδ inhibitors.

These lines will be tested for susceptibility to PKCδ knockdown by siRNA, or PKCδ inhibition by a small molecule inhibitor, with assay of cell numbers at 48 hrs by MTT assay.

TASK 3: Test the ability of PKCδ inhibitors to induce selective cytotoxicity in human prostate cancer stem cells.

Human prostate cancer stem cells (Oct 4, telomerase, SSEA 3/4 , and AP positive), and <u>normal</u> breast stem cells, are purchased from Celprogen (San Pedro, CA), cultured under conditions which maintain their undifferentiated state, and tested for their susceptibility to PKCδ knockdown by siRNA, or PKCδ inhibition by a small molecule inhibitor, with assay of cell numbers at 48 hrs by MTT assay

Status: IN PROGRESS

Progress:

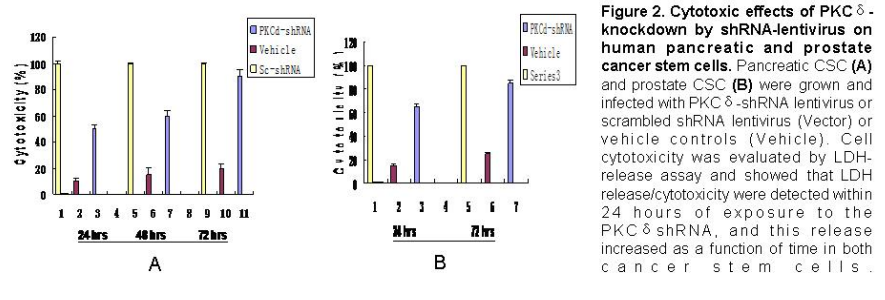

Figure 2. Cytotoxic effects of PKC δ-knockdown by shRNA-lentivirus on human pancreatic and prostate cancer stem cells. Pancreatic CSC **(A)** and prostate CSC **(B)** were grown and infected with PKC δ-shRNA lentivirus or scrambled shRNA lentivirus (Vector) or vehicle controls (Vehicle). Cell cytotoxicity was evaluated by LDH-release assay and showed that LDH release/cytotoxicity were detected within 24 hours of exposure to the PKC δ shRNA, and this release increased as a function of time in both cancer stem cells.

Fig. 23

We first demonstrated that prostate cancer stem cells (CSC) are susceptible to PKCδ suppression using siRNA (**Fig. 23, panel B**)

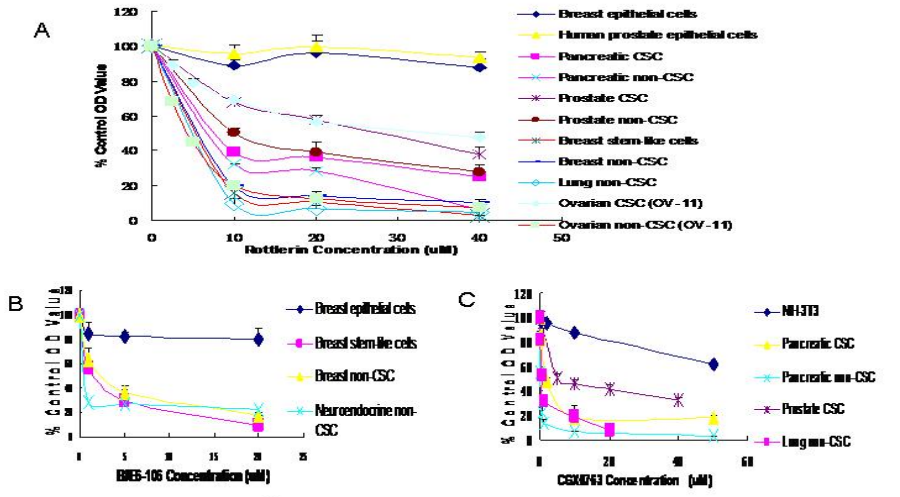

Figure 4. Effects of PKCδ inhibitors on proliferation of cancer stem cells and non-CSC tumor cell lines. Variety of non-CSC tumor cells and cancer stem cell or non-transformed human breast or prostate cells or NIH-3T3 cells were grown and exposed to Rottlerin **(A)** or BJE6-106 **(B)** or CGX0763 **(C)** at concentrations indicated. After 72 hours of exposure, cell growth was evaluated by MTT assay of viable cells and the growth curves showed that PKCδ inhibitors significantly inhibit proliferation of cancer cells and cancer stem cells but normal human and mouse cells.

Fig. 24

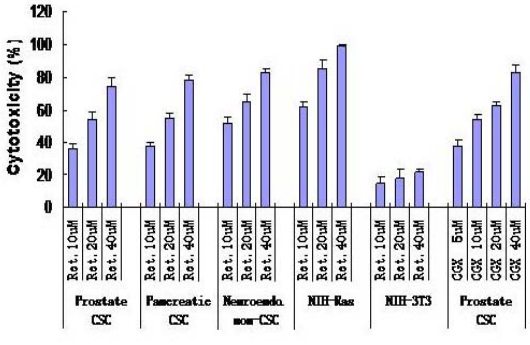

Figure 5. Cytotoxic effects of PKCδ-inhibitors on human tumor cells and cancer stem cells. Tumor cells and cancer stem cells or NIH and NIH-Ras cells were grown and exposed to Rottlerin or CGX0763 at concentrations indicated. After 72 hours of exposure, cell cytotoxicity was evaluated by LDH-release assay and showed that the cytotoxicity increased as a function of concentration of PKCδ inhibitors in tumor cells and cancer stem cells and NIH-Ras cells but normal cells.

Fig. 25

We then tested LC-1 and 2nd generation PKCδ-inhibitory compounds on prostate cancer CSCs.
Examples of such studies are shown below (**Figs. 24A&B, 25**)

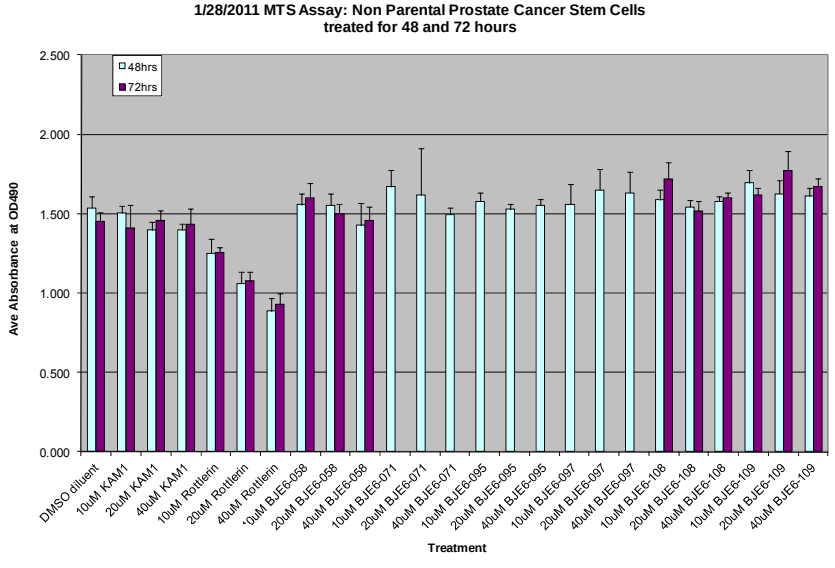

Fig. 26: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

We have also now tested the entire panel of 36 3rd generation PKCδ-inhibitory compounds against 2 prostate cancer stem cell cultures. Examples of such studies are shown below (**Figs. 26-36**)

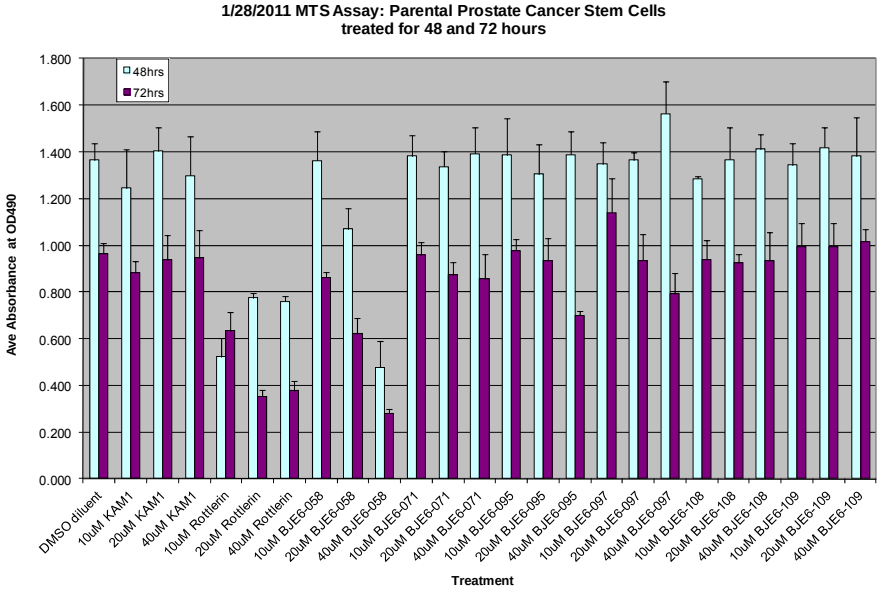

Fig. 27: Cell numb ass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

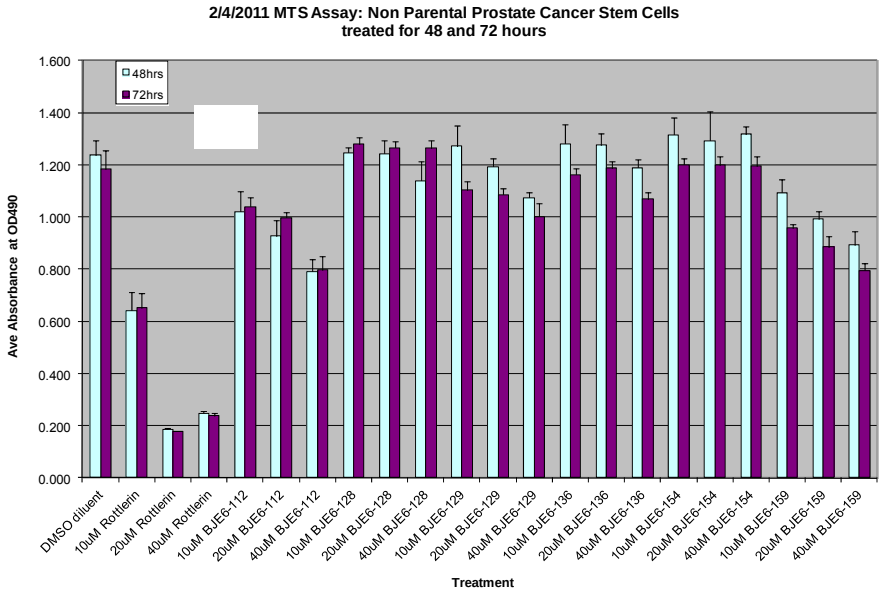

Fig. 28: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

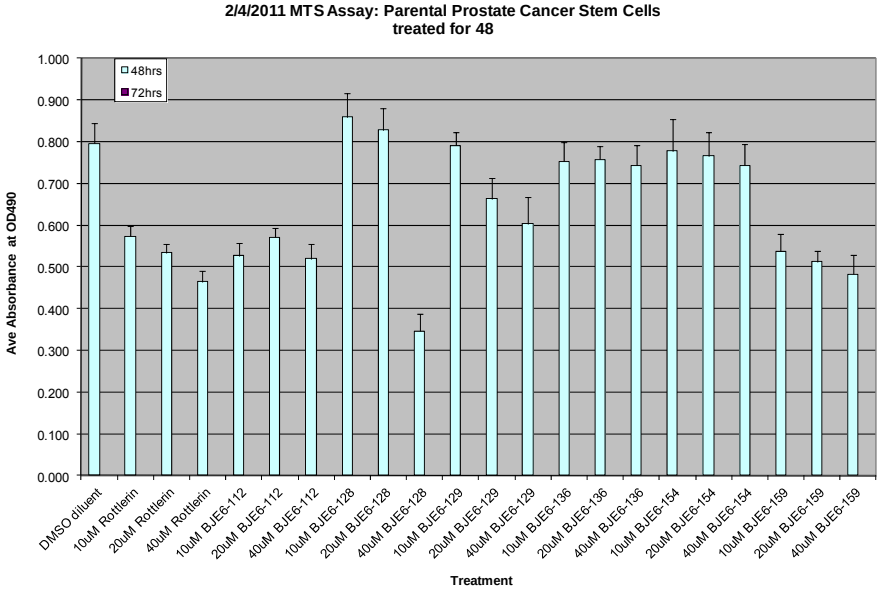

Fig. 29: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

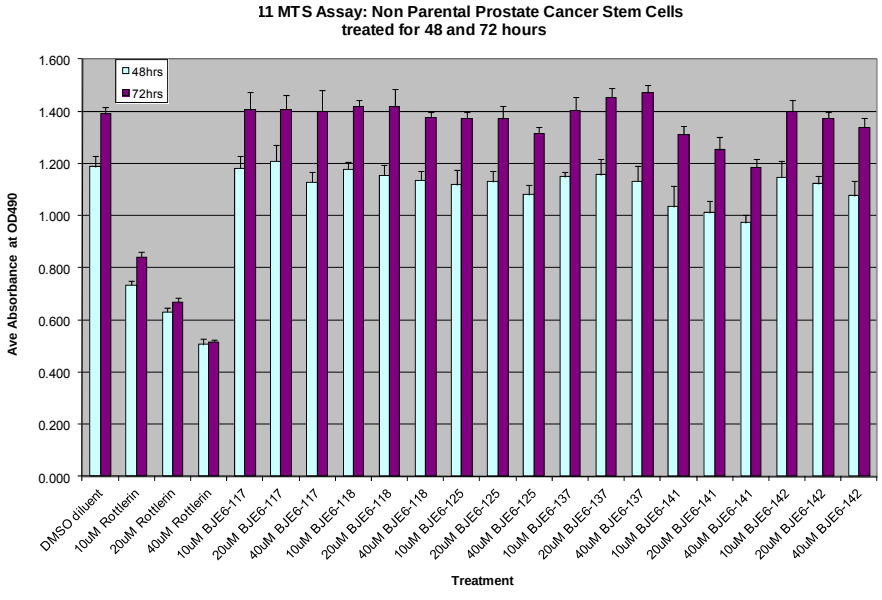

Fig. 30: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

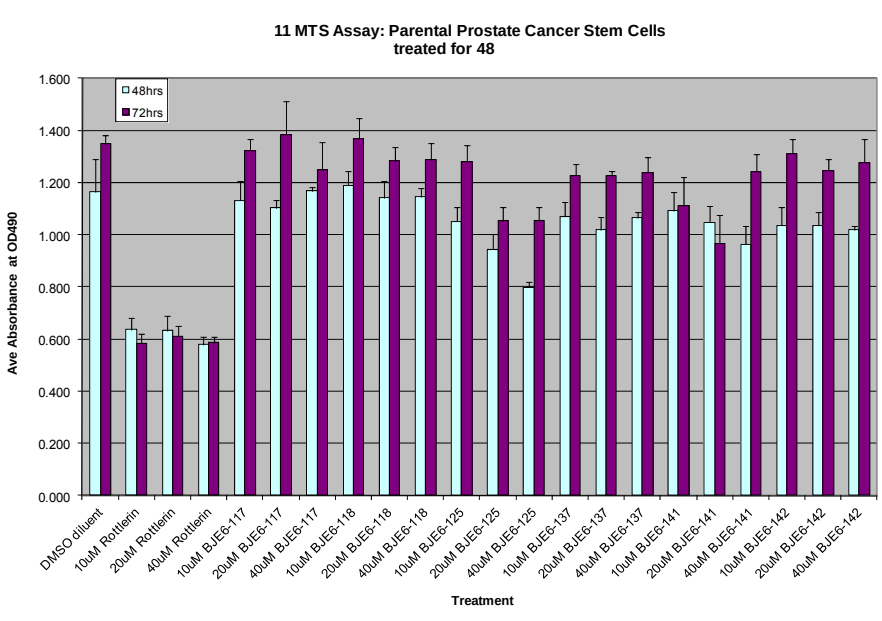

Fig. 31: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

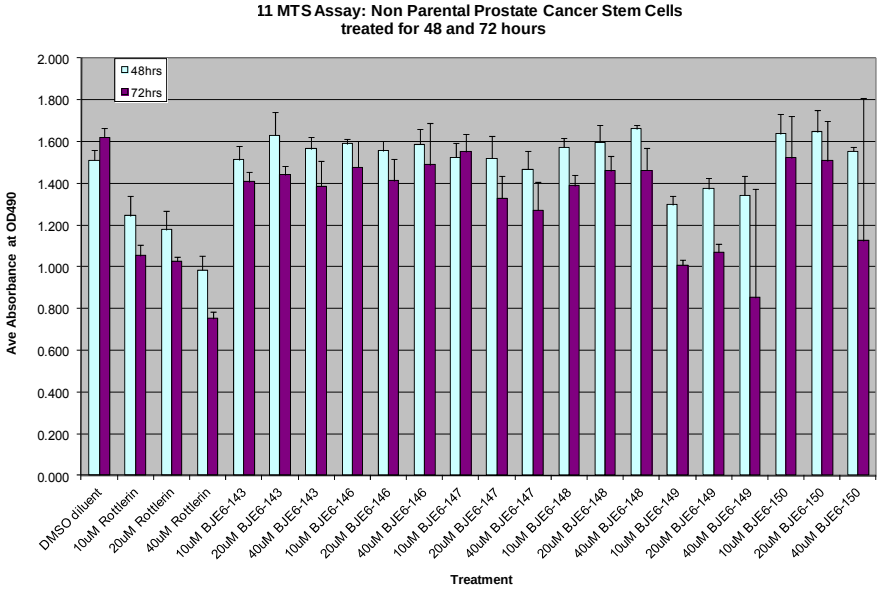

Fig. 32: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

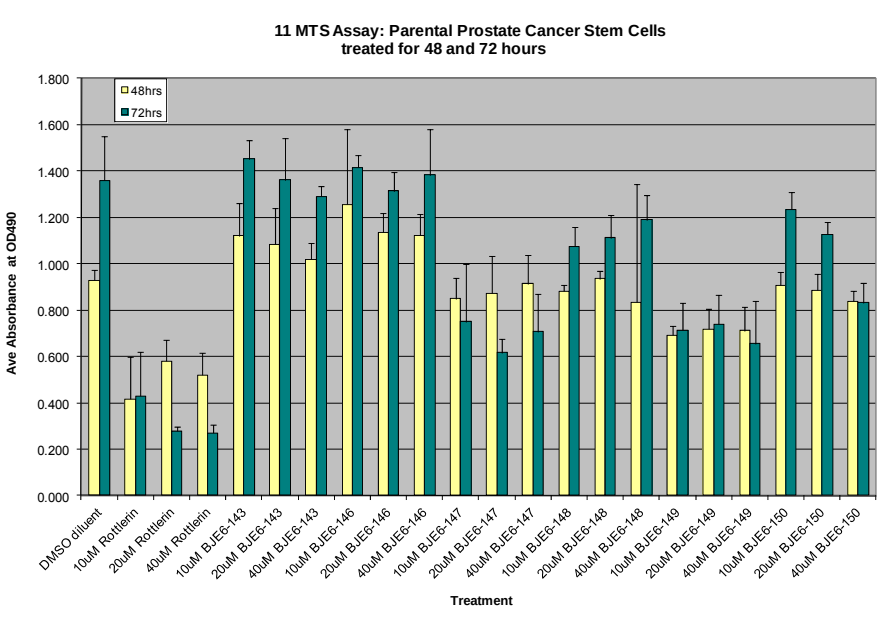

Fig. 33: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

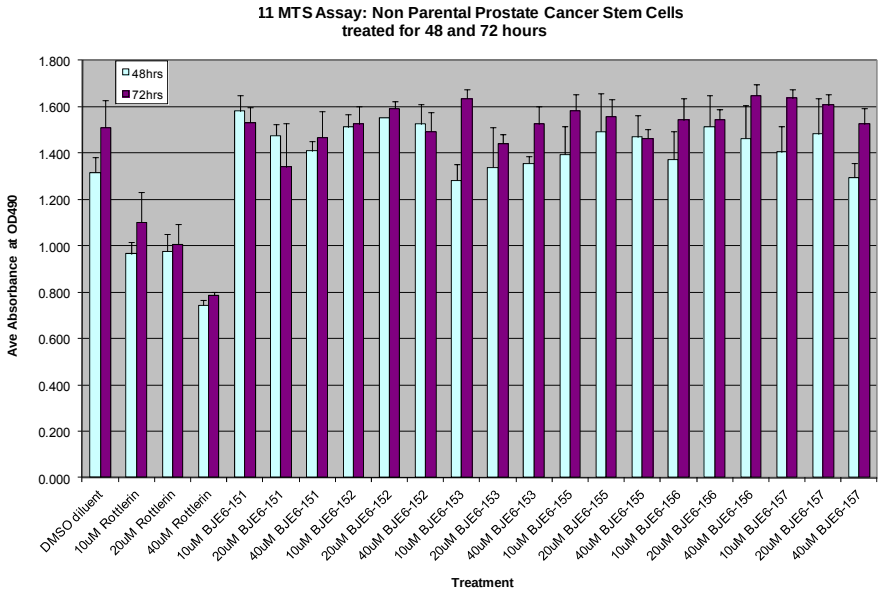

Fig. 34: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

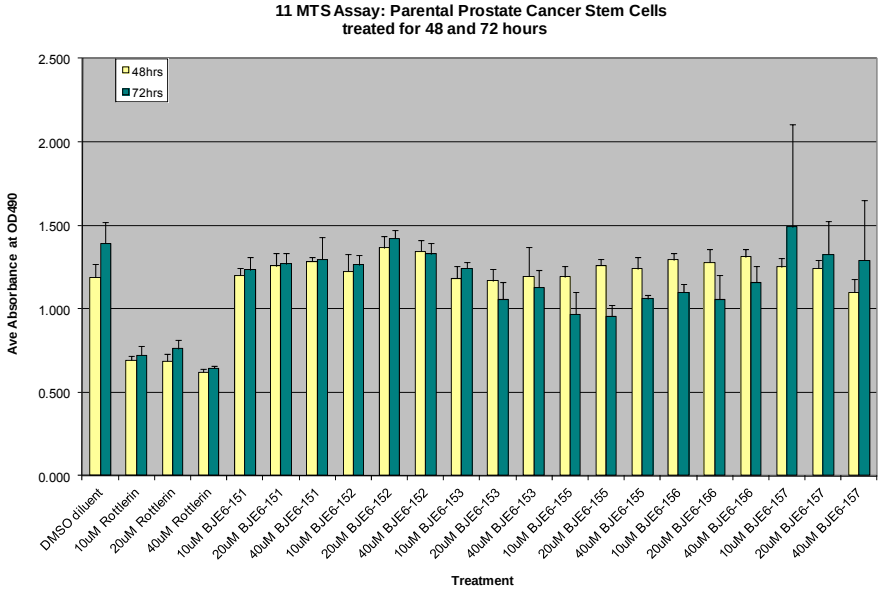

Fig. 35: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

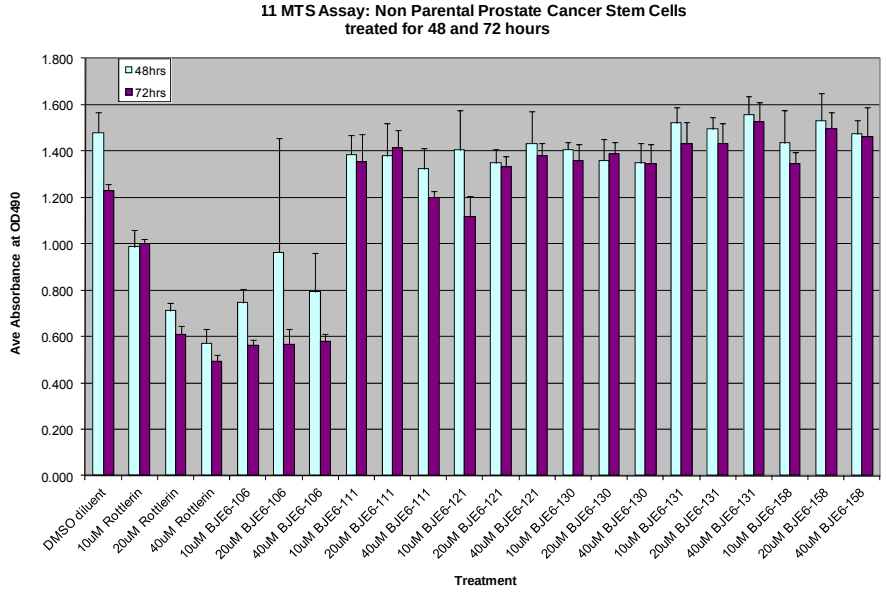

Fig. 36: Cell number (mass) at 48 or 72 h post exposure to the indicated compounds, expressed as OD490. Compounds tested at the concentrations indicated (μM).

Interpretation: Prostate cancer stem cell (CSC) lines are susceptible to PKCδ inhibition by siRNA or new small-molecule PKCδ inhibitors. Certain 3rd generation compounds (58, 106, 149, and 159) show toxicity against CSC lines comparable to LC-1 or greater than LC-1)

TASK 4: Test this Ras-targeted approach in an *in vivo* model of human prostate carcinoma.

Methods: Test this targeted approach in *in vivo* models of human prostate carcinoma. A xenograft model will be employed, utilizing an activating Ras-mutant human prostate carcinoma cell line (TSU-Pr-1) and a human prostate carcinoma cell line with aberrantly-activated PI_3K-signaling (PC3). In the last year, if time permits, a transgenic model of prostate cancer (the PB-Cre4 x PTEN(loxP/loxP) mouse), will be tested for sensitivity to this targeted therapeutic approach. Three cohorts of 15 immunodeficient (nu/nu) mice each, one vehicle control and two with different doses of the optimal PKC-δ inhibitor selected in TASK 1. Tumor growth will be serially quantitated.

Progress: This task has not yet been initiated, as we are waiting until the most potent small molecule inhibitors of PKCδ are identified prior to beginning these studies. We have established the MTD for B106, our lead compound at this time, and so are ready to initiate these studies.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Demonstrated the sensitivity human prostate cancers to PKCδ inhibition
- Showed activity of PKCδ inhibition against human prostate cancer stem cells
- Designed and synthesized 36 new compounds as PKCδ inhibitors
- Tested the activity of these 36 new compounds against PKCδ and PKCα
- Tested the activity of these 36 new compounds against human prostate cancer cells
- Tested the activity of these 36 new compounds against human prostate cancer *stem* cells
- Established MTD for our lead compound
- Determined the duration of exposure to PKCδ inhibitor drug necessary to achieve maximal cytotoxicity

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

None to date

CONCLUSION:

In our first year of work, we have made substantial progress. We have succeeded in demonstrating that multiple types of human prostate cancer cells are susceptible to PKCδ inhibition, using siRNA as a "specificity" test, and multiple structurally-distinct small molecule PKCδ inhibitors. These findings validate PKCδ as a target in prostate cancer, and provide proof-of-principle for the use of PKCδ inhibitors as potential therapeutics. Furthermore, we have shown the utility of PKCδ inhibition as a strategy for the elimination of prostate cancer stem cells. We have refined the initial PKCδ inhibitor lead compound now through 2 generations,

producing small molecules of increasing potency and PKCδ specificity. Our next generation will be optimized for "drug-like" properties, to facilitate moving into *in vivo* testing of tumor xenografts.

This *in vivo* testing in an animal will move these discoveries towards a medical product. Results of such studies will demonstrate the efficacy of this approach, provide informal toxicology, and informal PK.

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APPENDICES: None

SUPPORTING DATA: included above